Europäisches Patentamt | European Patent Office | Office européen des brevets

REC'D 22 NOV 2004
WIPO PCT

Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

03078308.8

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Offic

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

BEST AVAILABLE COPY

Anmeldung Nr:
Application no.: 03078308.8
Demande no:

Anmeldetag:
Date of filing: 16.10.03
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Applied Research Systems ARS Holding N.V.
Pietermaai 15
Curacao
ANTILLES NEERLANDAISES

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Therapeutic uses of chemokine variants

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

A61K38/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL PT RO SE SI SK TR LI

THERAPEUTIC USES OF CHEMOKINE VARIANTS

FIELD OF THE INVENTION

This patent describes novel therapeutic uses of chemokine variants, and in particular of human CCL2 variants.

BACKGROUND OF THE INVENTION

Chemokines are small, secreted pro-inflammatory proteins, which mediate directional migration of leukocytes from the blood to the site of injury. Depending on the position of the conserved cysteines characterizing this family of proteins, the chemokine family can be divided structurally in C, C-C, C-X-C and C-X_3-C chemokines, to which corresponds a series of membrane receptors (Baggiolini M et al., 1997; Fernandez EJ and Lolis E, 2002).

A series of membrane receptors, all heptahelical G-protein coupled receptors, are the binding partners that allow chemokines to exert their biological activity on the target cells, which may present specific combinations of receptors according to their state and/or type. The physiological effects of chemokines result from a complex and integrated system of concurrent interactions: the receptors often have overlapping ligand specificity, so that a single receptor can bind different chemokines, as well a single chemokine can bind different receptors.

Usually chemokines are produced at the site of injury and cause leukocyte migration and activation, playing a fundamental role in inflammatory, immune, homeostatic and angiogenic processes. Even though there are potential drawbacks in using chemokines as therapeutic agents (tendency to aggregate and promiscuous binding, in particular), these molecules, therefore, are considered good target candidates for therapeutic intervention in diseases associated to such processes, by

inhibiting specific chemokines and their receptors at the scope to preventing the excessive recruitment and activation of leukocytes (Baggiolini M, 2001; Loetscher P and Clark-Lewis I, 2001; Godessart N and Kunkel SL, 2001).

Studies on structure-activity relationships indicate that chemokines have two main sites of interaction with their receptors, the flexible amino-terminal region and the conformationally rigid loop that follows the second cysteine. Chemokines are thought to dock onto receptors by means of the loop region, and this contact is believed to facilitate the binding of the amino-terminal region that results in receptor activation. This importance of the amino-terminal region has been also demonstrated by testing natural and synthetic chemokines in which this domain is modified or shortened. This processing, following proteolytic digestion, mutagenesis, or chemical modification of amino acids, can either activate or render these molecules completely inactive, generating compounds with agonistic and/or antagonistic activity. Thus, chemokines with specific modifications in the amino-terminal region are considered having therapeutic potential for inflammatory and autoimmune diseases (Schwarz and Wells, 1999).

CCL2, also known as Monocyte Chemoattractant Protein 1 (MCP-1) or Monocyte Chemotactic And Activating Factor (MCAF), has been early identified as having central roles in inflammation, being capable of promoting the recruitment of monocytes and basophiles in response to injury and infection signals in various inflammatory diseases, different types of tumors, cardiac allograft, AIDS, and tuberculosis (Yoshimura T et al., 1989; Gu L et al., 1999). The physiological activities associated to CCL2 have been extensively studied by means of transgenic animals, which allowed to demonstrate that

also the expression of cytokines related to T helper responses and the initiation of atherosclerosis (Gu L et al., 2000; Gosling J et al, 1999; Lu B et al, 1998).

Structurally, CCL2 consists of a N-terminal loop and a beta sheet overlaid by an alpha helix at the C-terminal end, and forms homodimers (Handel T et al., 1996; Lubkowski J, et al., 1997). As for many other chemokines, the literature provides many examples of structure-activity studies in which CCL2 mutants have been generated by expressing N-terminal truncated or single site substituted variants to assess the role of the deleted or substituted amino acids in CCL2-associated binding activities and other properties (Gong J and Clark-Lewis I, 1995; Zhang Y et al., 1996; Steitz SA et al., 1998; Gu L et al., 1999; Hemmerich S et al., 1999; Seet BT et al., 2001).

In particular, the role of dimerization in CCL2 receptor binding and activation was studied showing that different mutations in the terminal region hindering dimerization may alter some CCL2 activities such as receptor binding affinity, stimulation of chemotaxis, inhibition of adenylate cyclase, and stimulation of calcium influx (Paavola C et al, 1998). While one mutant, herein called P8A*, does not dimerize, but maintains original potency and efficacy, another monomeric mutant, herein called Y13A*, has a 100-fold weaker binding affinity, is a much less potent inhibitor of adenylate cyclase and stimulator of calcium influx, and is unable to stimulate chemotaxis in cell culture. Finally, a mutant, [1+9-76]MCP-1 (a CCL2 variant lacking residues 2-8), antagonizes CCL2 *in vitro* activities.

However, there is not indication in the prior art that a specific chemokine mutant, and in particular a CCL2 mutant, being an obligate monomer due to a single site substitution, may act as a chemokine antagonist.

SUMMARY OF THE INVENTION

It has been surprisingly found that variants of homodimer-forming chemokines, such as CCL2, having a single amino acid substitution in the dimerization interface that alters the pattern of hydrogen bonds and acting as an obligate monomer, can antagonize natural chemokines and have anti-inflammatory activity *in vivo*. These variants can be used as active ingredient in pharmaceutical compositions, in particular for the treatment of inflammatory, autoimmune, or infectious diseases . Other features and advantages of the invention will be apparent from the following detailed description.

DESCRIPTION OF THE FIGURES

Figure 1: amino acid sequences of human and mutated CCL2 proteins on the basis of mature forms of human CCL2 used in the Examples (residue 24-99 of SWISSPROT Acc. N° P13500), having both a mutation in position 64 to Isoleucin (boxed residues; the relevant mutation P8A is underlined).

Figure 2: graph comparing the cell recruiting activity of recombinant human CCL2 and CCL2-P8A with the baseline in the peritoneal cell recruiting assay .

Figure 3: graph showing the dose-response inhibiting activity of CCL2-P8A in peritoneal cell recruitment assays using two different inducers: CCL2 (A) or Thioglycollate (B); the results are compared with the baseline level, a negative control (saline) and a positive control, Dexamethasone (Dex; only in B).

Figure 4: graph showing the inhibiting activity of CCL2-P8A in ovalbumin-induced

DETAILED DESCRIPTION OF THE INVENTION

In view of the above mentioned evidences in the prior art, there is no indication that a CCL2 variant, resulting from a single amino acid substitution and acting as obligate monomer, can antagonize *in vivo* CCL2, and in general can inhibit cell recruitment or inflammatory reactions.

Pharmaceutical uses, methods, and compositions that can be consequently envisaged for this specific monomeric variant of CCL2, called CCL2-P8A, can be also envisaged for any other monomeric variant of CCL2, or of other chemokines that are naturally active as dimers, that are generated in the same manner. These specific mutants should present antagonistic and inhibitory properties similar to the CCL2-P8A against the natural chemokine, and therefore they should have a medical applicability.

The main object of the present invention is the use of a monomeric variant of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface of said chemokine, as active ingredient in a pharmaceutical composition, in particular for the treatment or prevention of autoimmune, inflammatory, or infectious diseases.

Examples of dimer-forming chemokines are human CCL3, CCL3, and CXCL8, all chemokines known to be a therapeutic target for various diseases, such as autoimmune, inflammatory, or infectious diseases.

The monomeric variant should preferably result from a single amino acid substitution. More preferably, being proline an amino acid well known to be particularly relevant for establishing stereospecifc hydrogen bonds involved in the formation of protein complexes such as homodimers, the single amino acid substitution should be

the substitution of a proline with non-proline amino acid. Alternatively, the single amino acid substitution should be the substitution of non-proline amino acid with a proline.

An example of the above described substitution is monomeric variant of the mature form of CCL2 (SEQ ID NO: 1) called CCL2-P8A (SEQ ID NO: 2), wherein a proline known to be involved in CCL2 homodimerization (Paavola C et al, 1998), is substituted to an alanine, an amino acid having a different orientation of the chemical group possibly forming hydrogen bonds.

A further object of the present invention is the use of a monomeric variant of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface of said chemokine, such as CCL2-P8A, for the preparation of a pharmaceutical composition.

Alternative forms of the monomeric variants of the homodimer-forming chemokines above defined that can be used as active ingredients in pharmaceutical compositions include:

a) their active mutants;

b) polypeptides comprising them, or their active mutants, and an amino acid sequence belonging to a protein sequence other than said chemokine;

c) active fractions, precursors, salts, derivatives, complex or conjugate of (a) or (b).

These alternative compounds are intended to comprehend molecules with changes to the sequence of the monomeric variants of the homodimer-forming chemokines which do not affect the basic characteristics disclosed in the present

The term "active" means that such alternative compounds should maintain the functional features of the CCL2 mutants of the present invention, and should be as well pharmaceutically acceptable and useful.

The antagonistic properties of the monomeric variants of homodimer-forming chemoines defined above, and exemplified in the present patent application using CCL-P8A as CCL2 antagonist, can be maintained, or even potentiated, in the active mutants. This category of molecules includes natural or synthetic analogs of said sequence, wherein one or more amino acid residues have been added, deleted, or substituted, provided they display the same biological activity characterized in the present invention at comparable or higher levels, as determined by means known in the art and disclosed in the Examples below.

Natural analogs are intended the corresponding sequences of human chemokines proteins identified in other organisms, like mouse CCL2 (SWISSPROT Acc. N° P10148). Artificial analogs are intended peptides prepared by known chemical synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable thereof, which provide a finite set of substantially corresponding mutated or shortened peptides or polypeptides which can be routinely obtained and tested by one of ordinary skill in the art using the teachings presented in the prior art and in the Examples of the present patent application.

Preferred changes in these active mutants are commonly known as "conservative" or "safe" substitutions, and involve non-basic residues. Conservative amino acid substitutions are those with amino acids having sufficiently similar chemical properties, in order to preserve the structure and the biological function of the molecule. It is clear that insertions and deletions of amino acids may also be made in the above defined sequences without altering their function, particularly if the insertions

or deletions only involve a few amino acids, e.g., under ten, and preferably under three, and do not remove or displace amino acids which are critical to the functional conformation of a protein or a peptide.

The literature provide many models on which the selection of conservative amino acids substitutions can be performed on the basis of statistical and physico-chemical studies on the sequence and/or the structure of natural protein (Rogov SI and Nekrasov AN, 2001). Protein design experiments have shown that the use of specific subsets of amino acids can produce foldable and active proteins, helping in the classification of amino acid "synonymous" substitutions which can be more easily accommodated in protein structure, and which can be used to detect functional and structural homologs and paralogs (Murphy LR et al., 2000). The synonymous amino acid groups and more preferred synonymous groups are those defined in Table I.

Still in accordance to the present invention, other alternative monomeric variants of homodimer-forming chemokines may result from conventional mutagenesis technique of the encoding DNA, from combinatorial technologies at the level of encoding DNA sequence (such as DNA shuffling, phage display/selection), or from computer-aided design studies, followed by the validation for the desired activities as described in the prior art and in the Examples below.

A further group of active mutants of the monomeric variants of the homodimer-forming chemokines defined above are peptide mimetics (also called peptidomimetics), in which the nature of peptide or polypeptide has been chemically modified at the level of amino acid side chains, of amino acid chirality, and/or of the peptide backbone. These alterations are intended to provide monomeric variants of the homodimer-

Many other modifications providing increased potency, prolonged activity, easiness of purification, and/or increased half-life are known in the art (WO 02/10195; Villain M et al., 2001). Preferred alternative, "synonymous" groups for amino acids included in peptide mimetics are those defined in Table II.

The techniques for the synthesis and the development of peptide mimetics, as well as non-peptide mimetics, are well known in the art (Hruby VJ and Balse PM, 2000; Golebiowski A et al., 2001). Various methodology for incorporating unnatural amino acids into proteins, using both *in vitro* and *in vivo* translation systems, to probe and/or improve protein structure and function are also disclosed in the literature (Dougherty DA, 2000).

The present patent application discloses the use of monomeric variants of the homodimer-forming chemokines, and their active mutants, as active ingredients in pharmaceutical compositions, as well as of proteins comprising their amino acid sequence and an amino acid sequence belonging to a protein sequence other than said chemokine. This heterologous latter sequence should provide additional properties without impairing significatively the pharmaceutical applicability. Examples of such additional properties are an easier purification procedure, a longer lasting half-life in body fluids, or extracellular localization. This latter feature is of particular importance for defining a specific group of fusion or chimeric proteins included in the above definition since it allows these monomeric variants to be localized in the space where not only where the isolation and purification of these peptides is facilitated, but also where CCL2 naturally interact with other molecules.

Additional protein sequences which can be used to generate alternative forms of these monomeric variants of homodimer-forming chemokines as defined above are the ones of extracellular domains of membrane-bound protein, immunoglobulin constant

region, multimerization domains, extracellular proteins, signal peptide-containing proteins, export signal-containing proteins. The choice of one or more of these sequences to be fused to the monomeric variant is functional to specific use of said agent. When the additional protein sequence, as in the case of the sequence of extracellular, export signal, or signal-peptide containing proteins, allows the monomeric variant to be secreted in the extracellular space, the agent can be more easily collected and purified from cultured cells in view of further processing or, alternatively, the cells can be directly used or administered.

The monomeric variants of homodimer-forming chemokines defined above can be also used in other preferred forms, for example as active fractions, precursors, salts, derivatives, conjugates or complexes.

The term "fraction" refers to any fragment of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example residues of sugars or phosphates, or aggregates of the original polypeptide or peptide. Such molecules can result also from other modifications which do not normally alter primary sequence, for example *in vivo* or *in vitro* chemical derivativization of peptides (acetylation or carboxylation), those made by modifying the pattern of phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes) of a peptide during its synthesis and processing or in further processing steps.

The "precursors" are compounds which can be converted into the compounds of present invention by metabolic and enzymatic processing prior or after the

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the peptides, polypeptides, or analogs thereof, of the present invention. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids such as, for example, acetic acid or oxalic acid. Any of such salts should have substantially similar activity to the peptides and polypeptides of the invention or their analogs.

The term "derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the N- or C-terminal groups according to known methods. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups.

Conjugates or complexes of monomeric variants of homodimer-forming chemokines defined above can be generated, using molecules and methods known in the art of the interaction with receptor or other proteins (radioactive or fluorescent labels, biotin), therapeutic efficacy (cytotoxic agents), or improving the agents in terms of drug delivery efficacy, such as polyethylene glycol and other natural or synthetic polymers (Pillai O and Panchagnula R, 2001).

As a general procedure, the monomeric variants of homodimer-forming chemokines defined above can be produced may be prepared by any procedure known

in the art, including recombinant DNA-related technologies and chemical synthesis technologies.

Recombinant monomeric variants can be produced by generating nucleic acid segments encoding them, using common genetic engineering techniques, and cloning in replicable vector of viral or plasmid origin which are used to modify a prokaryotic or eukaryotic host cell, using episomal or non-/homologously integrated vectors, as well as transformation-, infection-, or transfection-based technologies. These vectors should allow the expression of the monomeric variant in the prokaryotic or eukaryotic host cell under the control of their own transcriptional initiation/termination regulatory sequences, which are chosen to be constitutively active or inducible in said cell. A cell line substantially enriched in such cells can be then isolated to provide a stable cell line.

Many books and reviews provides teachings on how to clone and produce recombinant proteins using vectors and prokaryotic or eukaryotic host cells, such as some titles in the series "A Practical Approach" published by Oxford University Press ("DNA Cloning 2: Expression Systems", 1995; "DNA Cloning 4: Mammalian Systems", 1996; "Protein Expression", 1999; "Protein Purification Techniques", 2001).

Examples of chemical synthesis technologies are solid phase synthesis and liquid phase synthesis. As a solid phase synthesis, for example, the amino acid corresponding to the C-terminus of the peptide to be synthetized is bound to a support which is insoluble in organic solvents, and by alternate repetition of reactions, one wherein amino acids with their amino groups and side chain functional groups protected with appropriate protective groups are condensed one by one in order from

chain is thus extended in this manner. Solid phase synthesis methods are largely classified by the tBoc method and the Fmoc method, depending on the type of protective group used. Typically used protective groups include tBoc (t-butoxycarbonyl), Cl-Z (2-chlorobenzyloxycarbonyl), Br-Z (2-bromobenzyloxycarbonyl), Bzl (benzyl), Fmoc (9-fluorenylmethoxycarbonyl), Mbh (4,4'-dimethoxydibenzhydryl), Mtr (4-methoxy-2,3,6-trimethylbenzenesulphonyl), Trt (trityl), Tos (tosyl), Z (benzyloxycarbonyl) and Cl2-Bzl (2,6-dichlorobenzyl) for the amino groups; NO2 (nitro) and Pmc (2,2,5,7,8-pentamethylchromane-6-sulphonyl) for the guanidino groups); and tBu (t-butyl) for the hydroxyl groups). After synthesis of the desired peptide, it is subjected to the de-protection reaction and cut out from the solid support. Such peptide cutting reaction may be carried with hydrogen fluoride or tri-fluoromethane sulfonic acid for the Boc method, and with TFA for the Fmoc method. Totally synthetic CCL2 proteins are disclosed in the literature (Brown A et al., 1996).

Purification of synthetic or recombinant monomeric variants of homodimer-forming chemokines defined above can be carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like. A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies or affinity groups, which bind the target protein and are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the column by heparin or by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength. Alternatively, HPLC (High Performance Liquid

Chromatography) can be used. The elution can be carried using a water-acetonitrile-based solvent commonly employed for protein purification.

Another object of the present invention is a monomeric variant of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface of said chemokine, such as CCL2-P8A, used as a medicament for autoimmune, inflammatory, or infectious diseases.

A further object of the present invention is a pharmaceutical composition containing a monomeric variant of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface of said chemokine, such as CCL2-P8A.

The pharmaceutical compositions of the invention may contain suitable pharmaceutically acceptable carriers, biologically compatible vehicles and additives that are suitable for administration to an animal (for example, physiological saline) and eventually comprising auxiliaries (like excipients, stabilizers or diluents) which facilitate the processing of the active compounds into preparations which can be used pharmaceutically. The pharmaceutical compositions may be formulated in any acceptable way to meet the needs of the mode of administration. For example, the use of biomaterials and other polymers for drug delivery, as well the different techniques and models to validate a specific mode of administration, are disclosed in literature (Luo B and Prestwich GD, 2001; Cleland JL et al., 2001).

"Pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with the effectiveness of the biological activity of the active ingredient and

injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

Any accepted mode of administration can be used and determined by those skilled in the art to establish the desired blood levels of the active ingredients. For example, administration may be by various parenteral routes such as subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. Parenteral administration can be by bolus injection or by gradual perfusion over time. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients known in the art, and can be prepared according to routine methods. In addition, suspension of the active compounds as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances increasing the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers. Pharmaceutical compositions include suitable solutions for administration by injection, and contain from about 0.01 to 99 percent, preferably from about 20 to 75 percent of active compound together with the excipient. Compositions that can be administered rectally include suppositories.

It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The dosage will be tailored to the individual subject, as is understood and determinable by one of skill in the art. The total dose required for each treatment may be administered by multiple doses or in a single

dose. The pharmaceutical composition of the present invention may be administered alone or in conjunction with other therapeutics directed to the condition, or directed to other symptoms of the condition. Usually a daily dosage of active ingredient is comprised between 0.01 to 100 milligrams per kilogram of body weight. Ordinarily 1 to 40 milligrams per kilogram per day given in divided doses or in sustained release form is effective to obtain the desired results. Second or subsequent administrations can be performed at a dosage, which is the same, less than, or greater than the initial or previous dose administered to the individual.

Another object of the present invention is a method for treating or preventing autoimmune, inflammatory, or infectious diseases comprising the administration of an effective amount of a monomeric variants of homodimer-forming chemokines, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface of said chemokine, such as CCL2-P8A.

An "effective amount" refers to an amount of the active ingredients that is sufficient to affect the course and the severity of the disease, leading to the reduction or remission of such pathology. The effective amount will depend on the route of administration and the condition of the patient.

Non-limitative examples of the autoimmune, inflammatory, or infectious diseases above indicated are the following: arthritis, rheumatoid arthritis (RA), psoriatic arthritis, osteoarthritis, systemic lupus erythematosus (SLE), systemic sclerosis, scleroderma, polymyositis, glomerulonephritis, fibrosis, fibrosis, allergic or hypersensitivity diseases, dermatitis, asthma, chronic obstructive pulmonary disease (COPD), inflammatory bowel disease (IBD), Crohn's diseases, ulcerative colitis,

The therapeutic applications of the polypeptides of the invention and of the related reagents can be evaluated (in terms or safety, pharmacokinetics and efficacy) by the means of the *in vivo* or *in vitro* assays making use of animal cell, tissues and models (Coleman RA et al., 2001; Li AP, 2001; Methods Mol. Biol vol. 138, "Chemokines Protocols", edited by Proudfoot A et al., Humana Press Inc., 2000; Methods Enzymol, vol. 287 and 288, Academic Press, 1997).

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions, which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

EXAMPLES

Example 1: construction and characterization of CCL2-P8A

Materials and methods

Expression of CCL2 and of CCL2-P8A

CCL2/MCP-1 (fig. 1; SEQ ID NO: 1) and the MCP-1/CCL2-P8A mutant protein (fig. 1; SEQ ID NO: 2) were generated and expressed in *E.coli* as described in the literature (Paavola CD et al, 1998) on the basis of the sequence of the mature form of human CCL2, corresponding to the segment 24-99 of the precursor molecule (SWISSPROT Acc. N° P13500), including the indicated substitution with an isoleucine in position 64.

All constructs were obtained and controlled by standard molecular biology technologies (PCR mutagenesis and amplification, DNA sequencing, restriction digestion).

Peritoneal cellular recruitment

Female Balb/C mice (Janvier, France) of 8 to 12 weeks were housed under normal animal holding conditions with a standard 12-h light/dark cycle and free access to food and water.

Groups composed of 3-6 mice were injected intraperitoneally with 200 µl of saline (sterile LPS-free NaCl 0.9% (w/v) or of this solution containing of CXCL8 or of one its mutants at 10 µg per injection.

For studies investigating the inhibitory effects of CCL2-P8A on CCL2-induced peritoneal cell recruitment, these molecules were administered intraperitoneally 30 minutes before the intraperitoneal injection of CXCL8. All the molecules were administered at the concentration and in buffer above indicated.

The assay for thioglycollate-induced peritoneal cell recruitment has been published (Mishell B, 1980). Briefly, thioglycollate medium was prepared by suspending 30 g of dehydrated thioglycollate medium (Becton Dickinson) in 1 liter of cold distilled water, then heated until boiling to dissolve the powder completely. The medium was then aliquoted into 100 ml bottles and autoclaved. After cooling, the medium was stored in the dark at room temperature for at least one month. Cellular recruitment was induced by intraperitoneal injection of mice in groups of 3 with 200 µl of a 3% solution of thioglycollate on Day 1, 30 minutes after CCL2-P8A administration. CCL2 was administered daily thereafter for 3 days (Days 2, 3 and 4). Dexamethasone (Sigma)

The ovalbumin-induced lung inflammation model was performed as published (Blyth *et al.*, 1996). Groups of 6 mice were sensitised by an intraperitoneal injection of 10 µg chicken egg albumin precipitated in 2 mg aluminium hydroxide 2% (Serva) in a total volume of 200 µl, which were prepared by mixing 25 µl ovalbumin (2mg/ml), 250 µl aluminium hydroxide in 725 µl LPS-free 0.9% NaCl and precipitated 3-4 hours at 4°C. Fifteeen days after sensitisation, mice were treated and challenged in groups of 6 mice with the intranasal administration of 15 µg ovalbumin in 50 µl saline, under inhaled anaesthesia (Isoflurane) daily from day 15 to 19. CCL2-P8A (200 µl, 10 µg per intraperitoneal injection) was administered 30 minutes before each challenge.

Peritoneal lavages to assess cell recruitment were performed at 4 hours after the CCL2 or CCL2-P8A final injection as follows. Mice were sacrificed by asphyxiation with rising concentrations of CO2 in a plexiglass box. Skin was cleaned with 70% ethanol. The outer layer of skin was removed, exposing the peritoneal membrane. The peritoneal cavity was lavaged 3 times with 5 ml ice cold PBS and fluid was pooled in a 15 ml polystyrene Falcon tube (Becton Dickinson) on ice. Each lavage was accompanied with a light massage of the peritoneal cavity. Lavage fluid was centrifuged at 425xg, the supernatant discarded and the resultant cell pellet was resuspended by gentle multiple pipetting in 1 ml PBS. 10 µl cell suspension was stained with 90 µl trypan blue and total cell counts were enumerated with a Neubauer haemocytometer by counting 4 areas each of 1 mm^2. The mean of the 4 counts was taken, multiplied by the dilution factor of 10, and multiplied again by 10 to give the number of cells per µl, according to the directions for use accompanying the haemocytometer. Finally the total value was multiplied by 1000 (to equal 1 ml) to arrive at the total cell number recovered.

Results

The obligate monomer, CCL2/MIP-1 mutant called CCL2-P8A was expressed in *E coli* (Figure 1) as described in the literature (Paavola CD et al, 1998), wherein only the *in vitro* properties were tested as being retained.

However, this monomeric form of CCL2 presents specific properties in *in vivo* assays. In the peritoneal cell recruitment assay, CCL-P8A is unable to recruit cells compared with natural CCL2 (Figure 2). Moreover, this molecule is able, in a dose dependent manner, to inhibit CCL2-induced (Figure 3A) and thioglycollate-induced macrophage recruitment (Figure 3B). In the latter assay, CCL2-P8A appears as effective as the positive control (dexamethasone, a known anti-inflammatory compound).

CCL2-P8A was also tested in a disease model, the ovalbumin-induced lung inflammation. In this classic model for allergic lung inflammation, the mice are sensitised with ovalbumin, with an adjuvant of aluminium hydroxide during sensitisation phase to boost the immune response, and then challenged by intranasal administration of ovalbumin over a period of 5 consecutive days, wherein CCL2-P8A was administered intraperitoneally throughout this phase. Also in this case, CCL2-P8A was capable to inhibit cell recruitment (figure 4).

Therefore, monomeric variant of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface, are shown to be inhibitors of chemokine-mediated cell recruitment in *in vivo* cell recruitment assays, implying that this can be a novel strategy for generating chemokine variants which can be used for preparing pharmaceutical compositions and therapeutic methods.

TABLE I

Amino Acid	Synonymous Group	More Preferred Synonymous Groups
Ser	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Arg	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Leu	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Pro	Gly, Ala, Ser, Thr, Pro	Pro
Thr	Gly, Ala, Ser, Thr, Pro	Thr, Ser
Ala	Gly, Thr, Pro, Ala, Ser	Gly, Ala
Val	Met, Phe, Ile, Leu, Val	Met, Ile, Val, Leu
Gly	Ala, Thr, Pro, Ser, Gly	Gly, Ala
Ile	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Phe	Trp, Phe,Tyr	Tyr, Phe
Tyr	Trp, Phe,Tyr	Phe, Tyr
Cys	Ser, Thr, Cys	Cys
His	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Gln	Glu, Asn, Asp, Gln	Asn, Gln
Asn	Glu, Asn, Asp, Gln	Asn, Gln
Lys	Asn, Lys, Gln, Arg, His	Arg, Lys, His
Asp	Glu, Asn, Asp, Gln	Asp, Glu
Glu	Glu, Asn, Asp, Gln	Asp, Glu
Met	Phe, Ile, Val, Leu, Met	Ile, Val, Leu, Met
Trp	Trp, Phe,Tyr	Trp

5

TABLE II

Amino Acid	Synonymous Group
Ser	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Arg	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-.Met, D-Ile, Om, D-Om
Leu	D-Leu, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Pro	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Thr	D-Thr, Ser, D-Ser, allo-Thr, Met,D-Met, Met(O), D-Met(O), Val, D-Val
Ala	D-Ala, Gly, Aib, B-Ala, Acp, L-Cys, D-Cys
Val	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met, AdaA, AdaG
Gly	Ala, D-Ala, Pro, D-Pro, Aib, .beta.-Ala, Acp
Ile	D-Ile, Val, D-Val, AdaA, AdaG, Leu, D-Leu, Met, D-Met
Phe	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, AdaA, AdaG, cis-3,4, or 5-phenylproline, Bpa, D-Bpa
Tyr	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Cys	D-Cys, S--Me--Cys, Met, D-Met, Thr, D-Thr
Gln	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Asn	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Lys	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Asp	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Glu	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Met	D-Met, S--Me--Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val

REFERENCES

Baggiolini M et al., Annu Rev Immunol, 15: 675-705, 1997.

Baggiolini M, J Intern Med, 250: 91-104, 2001.

Brown A et al., J Pept Sci 2 :40-46, 1996.

Cleland JL et al., Curr Opin Biotechnol,12: 212-9, 2001.

Dougherty DA, Curr Opin Chem Bio, 4: 645-52, 2000.

Fernandez EJ and Lolis E, Annu Rev Pharmacol Toxicol, 42:469-499, 2002.

Godessart N and Kunkel SL, Curr Opin Immunol, 13: 670-675, 2001.

Golebiowski A et al., Curr Opin Drug Discov Devel, 4: 428-34, 2001.

Gong J and Clark-Lewis I, J Exp Med 181: 631-640, 1995.

Gosling J et al., J Clin Invest, 103: 773-778, 1999.

Gu L et al., Chem Immunol, 72: 7-29, 1999.

Gu L et al., Nature, 404: 407-411, 2000.

Handel T et al., Biochemistry,35 : 6569-6584, 1996.

Hemmerich S et al., Biochemistry, 38: 13013-13025, 1999.

Hruby VJ and Balse PM, Curr Med Chem, 7:945-70, 2000

Loetscher P and Clark-Lewis I, J Leukoc Biol, 69: 881-884, 2001.

Lu B et al., J Exp Med, 187: 601-608, 1998.

Lubkowski J et al., Nat Struct Biol, 4: 64-69, 1997.

Luo B and Prestwich GD, Exp Opin Ther Patents, 11: 1395-1410, 2001.

Mishell B, Immunopharm, 2: 233-45. 1980.

Murphy LR et al., Protein Eng, 13:149-52, 2000.

Paavola C et al., J Biol Chem, 273: 33157-33165, 1998.

Pillai O and Panchagnula R, Curr Opin Chem Biol, 5: 447-451, 2001.

Rogov SI and Nekrasov AN, Protein Eng, 14: 459-463, 2001.

Schwarz MK and Wells TN, Curr Opin Chem Biol, 3: 407-17, 1999.

Seet BT et al., Proc Natl Acad Sci U S A, 98: 9008-9013, 2001.

Steitz SA et al., FEBS Lett , 430: 158-164, 1998.

Villain M et al., Chem Biol, 8: 673-9, 2001.

Yoshimura T et al., FEBS Lett, 244: 487-93, 1989.

Zhang Y et al., Methods, 10: 93-103, 1996.

CLAIMS

1. The use of a monomeric variant of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface of said chemokine, as active ingredient in a pharmaceutical composition.
2. The use of claim 1 wherein the pharmaceutical composition is for the treatment or prevention of autoimmune, inflammatory, or infectious diseases.
3. The use of claim 1 or 2 wherein the homodimer-forming chemokine is human CCL2, CCL3, or CXCL8.
4. The use of any of the claims from 1 to 3 wherein the monomeric variant result from a single amino acid substitution.
5. The use of claim 4 wherein the single amino acid substitution is the substitution of a proline with non-proline amino acid.
6. The use of claim 4 wherein the single amino acid substitution is the substitution of non-proline amino acid with a proline.
7. The use of claim 5 wherein the monomeric variant is CCL2-P8A (SEQ ID NO: 2).
8. The use of any of the claims from 1 to 7, wherein the monomeric variants of the homodimer-forming chemokines is in the form of :
   a) an active mutant;
   b) a polypeptides comprising said variant, or said active mutant, and an amino acid sequence belonging to a protein sequence other than said chemokine;
   c) an active fraction, precursors, salt, derivative, complex or conjugate of (a) or (b).
9. The use of a monomeric variant of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern

of hydrogen bonds at the dimerization interface of said chemokine, for the preparation of a pharmaceutical composition.

10. The use of claim 9, wherein said variant is CCL2-P8A (SEQ ID NO: 2).

11. A monomeric variant of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface of said chemokine, used as a medicament for autoimmune, inflammatory, or infectious diseases.

12. The monomeric variant of claim 11, wherein said variant is CCL2-P8A (SEQ ID NO: 2).

13. The pharmaceutical composition comprising a monomeric variant of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface of said chemokine.

14. The pharmaceutical composition of claim 13 wherein the monomeric variant is CCL2-P8A (SEQ ID NO: 2).

15. A method for treating or preventing autoimmune, inflammatory, or infectious diseases comprising the administration of an effective amount of a monomeric variants of a homodimer-forming chemokine, wherein said variant result from at least an amino acid substitution that alters the pattern of hydrogen bonds at the dimerization interface of said chemokine.

16. The method of claim 15 wherein the monomeric variant is CCL2-P8A (SEQ ID NO: 2).

ABSTRACT

Variants of homodimer-forming chemokines, such as CCL2, having a single amino acid substitution in the dimerization interface that alters the pattern of hydrogen bonds and acting as an obligate monomer, can antagonize natural chemokines and have anti-inflammatory activity *in vivo*. These variants can be used as active ingredient in pharmaceutical compositions for the treatment of inflammatory, autoimmune, or infectious diseases.

Figure 1

```
CCL2       1 QPDAINAPVT CCYNFTNRKI SVQRLASYRR ITSSKCPKEA VIFKTIVAKE  50
CCL2-P8A   1 QPDAINAAVT CCYNFTNRKI SVQRLASYRR ITSSKCPKEA VIFKTIVAKE  50

CCL2      51 ICADPKQKWV QDSMDHLDKQ TQTPKT 76
CCL2-P8A  51 ICADPKQKWV QDSMDHLDKQ TQTPKT 76
```

Figure 2

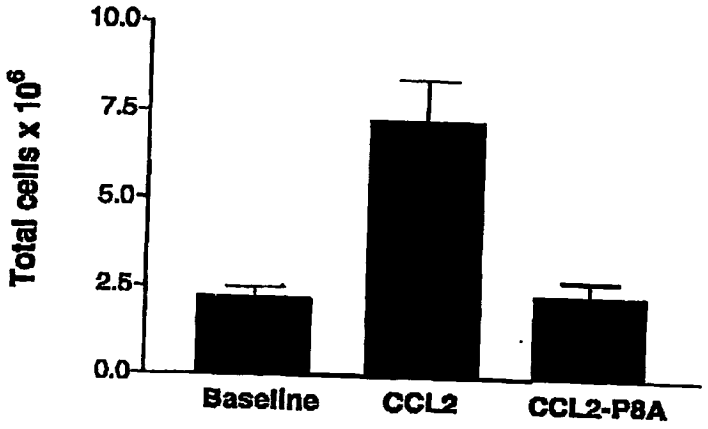
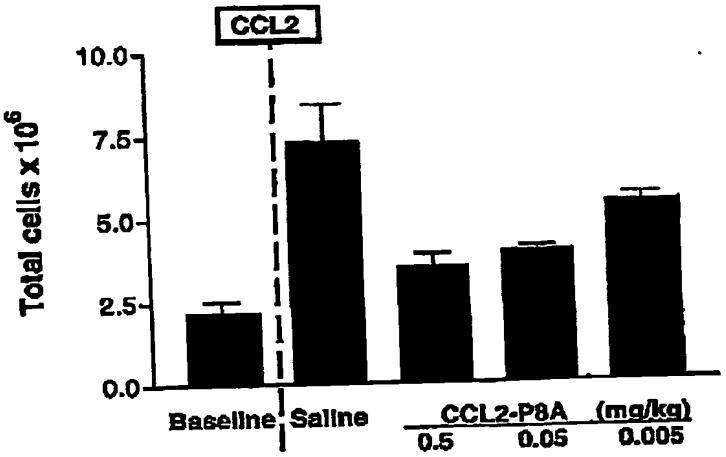


Figure 3

A)


CCL2
Total cells x 10^6
10.0
7.5
5.0
2.5
0.0
Baseline
Saline
CCL2-P8A (mg/kg)
0.5
0.05
0.005

B)

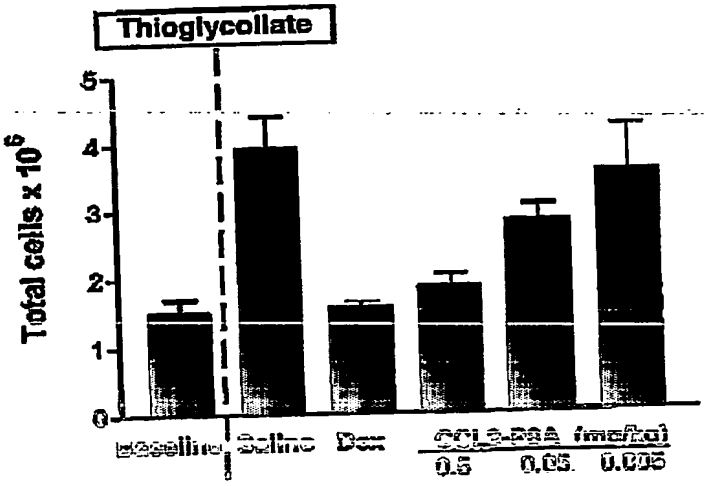
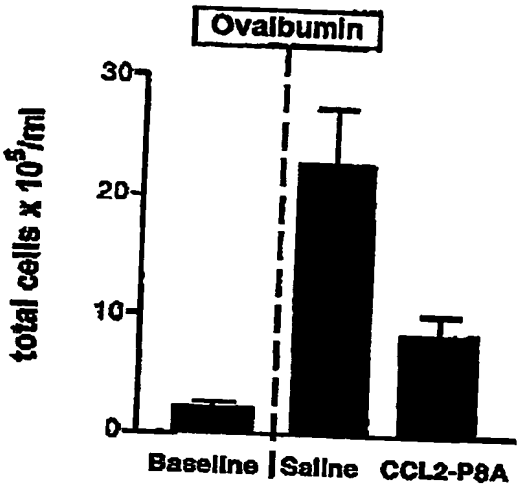

Thioglycollate
Total cells x 10^6
5
4
3
2
1
0
Baseline
Saline
Dex
0.5
0.05
0.005

Figure 4


Ovalbumin
30
20
10
0
total cells x 10⁶/ml
Baseline
Saline
CCL2-P8A

0933Z-seq
SEQUENCE LISTING

<110> Applied Research Systems ARS holding N.V.

<120> THERAPEUTIC USES OF CHEMOKINE VARIANTS

<130> 933 EP/EPA

<160> 2

<170> PatentIn version 3.0

<210> 1
<211> 76
<212> PRT
<213> Homo sapiens

<400> 1

Gln Pro Asp Ala Ile Asn Ala Pro Val Thr Cys Cys Tyr Asn Phe Thr
1 5 10 15

Asn Arg Lys Ile Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile Thr
20 25 30

Ser Ser Lys Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Ile Val Ala
35 40 45

Lys Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val Gln Asp Ser Ile
50 55 60

Asp His Leu Asp Lys Gln Thr Gln Thr Pro Lys Thr
65 70 75

<210> 2
<211> 76
<212> PRT
<213> synthetic construct

<400> 2

Gln Pro Asp Ala Ile Asn Ala Ala Val Thr Cys Cys Tyr Asn Phe Thr
1 5 10 15

Asn Arg Lys Ile Ser Val Gln Arg Leu Ala Ser Tyr Arg Arg Ile Thr
20 25 30

Ser Ser Lys Cys Pro Lys Glu Ala Val Ile Phe Lys Thr Ile Val Ala
35 40 45

Lys Glu Ile Cys Ala Asp Pro Lys Gln Lys Trp Val Gln Asp Ser Ile
50 55 60

Asp His Leu Asp Lys Gln Thr Gln Thr Pro Lys Thr
65 70 75

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.